

Myeloma Cells Are Highly Sensitive to the Granule Exocytosis Pathway Mediated by WT1-Specific Cytotoxic T Lymphocytes

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ABSTRACT

Purpose: Because WT1 is a universal tumor antigen, we examined the sensitivity of myeloma cells to WT1-specific cytotoxic T lymphocyte (CTL)-mediated cytotoxicity.

Experimental Design: WT1 expression in hematologic malignant cells was examined by quantitative reverse transcription-polymerase chain reaction. The cytotoxicity of a WT1-specific CTL clone against hematologic malignant cells, including myeloma cells, was examined by standard chromium-51 release assays. The extent of membrane damage induced by purified perforin was examined. Induction of WT1-specific CTLs from the patients with multiple myeloma (MM) was attempted, and we examined their function against myeloma cells.

Results: The expression levels of WT1 mRNA in myeloma and lymphoma cells were significantly lower than that in acute leukemia cells. Although the WT1 expression levels in myeloma and lymphoma cells were almost same, only myeloma cells were lysed efficiently by WT1-specific CTLs in a HLA-restricted manner. The amounts of interferon- γ produced by WT1-specific CTLs in response to stimulation with myeloma cells and with lymphoma cells were almost the same, suggesting that WT1 protein is processed and

expressed in the context of HLA class I molecules similarly on both myeloma and lymphoma cells. The extent of membrane damage induced by purified perforin appeared to be significantly higher in myeloma cells than in lymphoma cells. WT1-specific CTLs appeared to be present in patients with MM.

Conclusions: The present study has shown that susceptibility of membranes to perforin is an important factor determining the sensitivity of target cells to CTL-mediated cytotoxicity and that WT1 is an ideal target antigen for cellular immunotherapy of MM.

INTRODUCTION

Multiple myeloma (MM) is a fatal hematologic malignancy characterized by monoclonal growth of plasma cells (1, 2). Although recent therapeutic approaches for MM, including high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation, have improved the overall survival rate, MM is still an incurable disease. Recently, treatment strategies targeting mechanisms whereby myeloma cells grow and survive in the bone marrow, including thalidomide and its potent immunomodulatory derivatives and the proteasome inhibitor bortezomib, have been developed and are expected to improve the outcome of patients with MM resistant to conventional treatment (3, 4); however, the therapeutic efficacy of these agents is limited. Because cancer immunotherapy is tumor specific and less toxic, it seems an ideal therapeutic strategy for MM. The identification of target antigens that are expressed preferentially in tumor cells but not in normal cells and are recognized by T lymphocytes is essential to the development of efficacious cellular immunotherapy; however, to date, only a limited number of MM-associated antigens that are recognized by T lymphocytes have been identified.

The *WT1* gene encodes a zinc finger transcription factor (5), and WT1 binds to the early growth response-1 DNA consensus sequence present in various growth factor gene promoters (6). Although WT1 was initially shown to act as a transcriptional repressor, the specific functions of WT1 in normal and neoplastic tissues remain to be fully elucidated. During normal ontogenesis, the *WT1* gene is expressed in a time- and tissue-dependent manner, mainly in the fetal kidney, testis, ovary, and supportive structures of mesodermal origin (7). In contrast, in adults, *WT1* gene expression is limited to very few tissues, including the splenic capsule and stroma, the Sertoli cells of the testis, the granulosa cells of the ovary, the podocytes of the kidney, and CD34⁺ hematopoietic progenitor cells (8-10). With regard to malignant cells, it has been reported that most cases of acute leukemia and blast crisis of chronic myelogenous leukemia aberrantly overexpress WT1 (11-15). Previous studies have shown that the expression level of WT1 in B-lymphoma cells is significantly lower than that in acute leukemia (16); however, the details of WT1 expression in MM and other types

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of mature B-cell malignancies have not been reported. In the present study, we addressed the question of whether WT1 is expressed abundantly in myeloma cells and whether WT1-specific cytotoxic T lymphocytes (CTLs) can exert cytotoxicity against myeloma cells in an antigen-specific and HLA-restricted manner.

Another question raised in the present study is the nature of the mechanisms determining the sensitivity of target cells to CTL-mediated cytotoxicity. The mechanisms of cytotoxicity mediated by CTLs have been examined extensively, mainly in murine systems using various mutant and knockout mice, and various pathways have been identified. Among these, the granule exocytosis pathway mediated by perforin/granzymes and the Fas/Fas ligand pathway are thought to be the main mechanisms of CTL-mediated antigen-specific cytotoxicity (17). We recently used a combination of Fas-deficient target cells and perforin-deficient effector T lymphocytes to show that the granule exocytosis pathway is important in antigen-specific cytotoxicity mediated by human CD4⁺ as well as CD8⁺ CTLs (18–20). CTLs lyse target cells via recognition of the complex of target antigen-derived peptide and HLA molecule. Therefore, the susceptibility of target cells to antigen-specific cytotoxicity mediated by CTLs is thought to depend primarily on their expression levels of target antigen and HLA molecules. In the present study, we examined the mechanisms of cytotoxicity against myeloma cells mediated by WT1-specific CTLs, focusing on the sensitivity of target cells to perforin-mediated cytotoxicity. The data obtained from the present series of experiments revealed that WT1 expression levels in myeloma cells and lymphoma cells were both significantly lower than that in acute leukemia cells; however, myeloma cells, but not lymphoma cells, were lysed efficiently by WT1-specific CTLs. The extent of membrane damage induced by purified perforin in myeloma cells appeared to be significantly higher than that induced in lymphoma cells. In addition, WT1-specific CTL precursors were detected in peripheral blood of the patients with MM. On the basis of the present data, we discuss the feasibility of targeting WT1 in cellular immunotherapy for MM.

MATERIALS AND METHODS

Cell Separation and Cell Lines. Bone marrow mononuclear cells were isolated from the patients with MM and healthy volunteers after obtaining informed consent and stored in liquid nitrogen until use. B-lymphoblastoid cell lines [B-(LCLs)] were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus. LCLs were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS). The *HLA-A*2402* gene-transfected T2 cell line (T2-A24) was cultured in RPMI 1640 supplemented with 10% FCS and 800 µg/mL Geneticin (Life Technologies, Inc., Rockville, MD). All of the leukemia, myeloma, and lymphoma cell lines were cultured in RPMI 1640 supplemented with 10% FCS. All lymphoma cell lines used in the present study were established from patients with B-cell diffuse large non-Hodgkin's lymphoma or Burkitt's lymphoma. After obtaining consent from parents, cord blood was collected, and mononuclear cells were separated by Ficoll-Conray density gradient centrifugation. CD34⁺ cells were isolated from cord blood mononuclear cells with immunomagnetic

beads (MACS beads; Miltenyi Biotec, Auburn, CA) coated with anti-CD34 monoclonal antibody (MoAb). Immunomagnetic separations were performed according to the manufacturer's instructions.

Generation of WT1 Peptide-Specific Cytotoxic T Lymphocytes. WT1 peptide-specific CTLs were generated as described below. Peripheral blood mononuclear cells isolated from five HLA-A24-positive MM patients and three healthy volunteers after obtaining informed consent were plated in 96-well round-bottomed plates at 1×10^5 cells per well in the presence of the WT1-derived peptide WT1-T2 (CMTWNQMNL, residues 235–243) at a concentration of 10 µmol/L in RPMI 1640 supplemented with 10% human AB-type serum, 5 ng/mL human recombinant interleukin (IL)-7 (Genzyme, Boston, MA), and 100 pg/mL human recombinant IL-12 (Genzyme). After culturing for 7 days, the cells were restimulated by adding autologous mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan)-treated peripheral blood mononuclear cells and the WT1-T2 peptide at 10 µmol/L. After an additional 7 days of culture, the cells were restimulated in the same way. The next day, IL-2 (Boehringer Mannheim, Mannheim, Germany) was added to a final concentration of 10 units/mL. Ten days after the final stimulation, the cells in 20 µL (about 2×10^4 T lymphocytes per well) in each culture well were tested by ELISPOT assays for their antigen specificity. For expansion of WT1 peptide-specific bulk CTLs, the cells that showed specific spots in ELISPOT assays were stimulated by adding MMC-treated T2-A24 cells and 10 µmol/L WT1-T2 peptide, and then the specificity and cytotoxicity of the growing cells were examined by detection of interferon (IFN)-γ production by enzyme-linked immunosorbent assay (ELISA; Endogen, Rockford, IL) and chromium-51 release assay, respectively.

Establishment of a WT1 Peptide-Specific Cytotoxic T Lymphocyte Clone. A CTL clone designated TAK-1, which specifically recognizes WT1-T2 peptide in the context of HLA-A*2402, was established as described previously (16, 21). Briefly, CD8⁺ T lymphocytes were stimulated repeatedly with WT1-T2 peptide-loaded dendritic cells. The cytotoxicity of the growing cells was examined, and cells that exerted a cytotoxic effect on a WT1-T2 peptide-loaded autologous B-LCL were cloned by a limiting dilution method as described previously (22).

Quantitative Analysis of WT1 Messenger RNA Expression. Total RNA was extracted from samples with an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed in a final volume of 50 µL with the One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The reaction, carried out with 0.1 µg of total RNA from each sample, was performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reverse transcription of the RNA was achieved at 48°C for 30 minutes, and polymerase chain reaction was performed with an enzyme activation step (10 minutes at 95°C) followed by 40 cycles of denaturation/annealing/extension (15 seconds at 95°C and 1 minute at 60°C). Sequences of primers and probes were as follows: *WT1* forward primer, 5'-CAAC-CACAGCACAGGGTACG-3'; *WT1* reverse primer, 5'-TCTG-

TATTGGGCTCCGCAG-3'; and probe, 5'-FAM-AGCGATA-ACCACACAACGCCCATCC-TAMRA-3'. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* quantitative analysis was performed with predeveloped TaqMan assay reagent target kits (Applied Biosystems). All analyses were performed in duplicate. To normalize differences in RNA degradation between the individual samples and in RNA loading for the RT-PCR procedure, the *WT1* expression level for a particular sample was defined as its *WT1* gene expression level divided by its *GAPDH* gene expression level (23). The *WT1* gene expression level of K562 leukemia cells, which strongly express *WT1*, was designated 1.0, and the levels for the experimental samples were calculated relative to this value (12).

Flow Cytometric Analysis. The expression levels of HLA class I molecules on myeloma and lymphoma cells were determined by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated anti-HLA-A, -B, -C MoAb (BD PharMingen, San Diego, CA). HLA-A24 expression on cells was examined by flow cytometry using an anti-HLA-A24 MoAb (One Lambda, Canoga Park, CA) with mouse IgG as the control. The stained cells were analyzed with a flow cytometer (FACScan; Becton Dickinson, San Jose, CA). Measurement of mean fluorescence intensity and analysis of data were done with Cell Quest Software (Becton Dickinson).

Purification of Perforin. Human perforin was purified as described previously (24, 25). Briefly, perforin was extracted in 1 mol/L NaCl from granules of the human natural killer (NK) cell line YT and purified by ion metal affinity chromatography with an imidazole gradient in 10% betaine (IMAC; PerSeptive Biosystems, Cambridge, MA). Fractions with hemolytic activity as determined by sheep red blood cell assays were concentrated by Centricon ultrafiltration and stored in 2 mmol/L EDTA and 0.1% fatty acid-free bovine serum albumin until use. Purification of perforin was confirmed by SDS-PAGE and Western blotting (24).

Cytotoxicity Assays. ^{51}Cr release assays were performed as described previously (26). Briefly, 1×10^4 ^{51}Cr ($\text{Na}_2^{51}\text{CrO}_4$; New England Nuclear, Boston, MA)-labeled target cells and various numbers of effector cells in 200 μL of RPMI 1640 supplemented with 10% FCS were seeded into round-bottomed microtiter wells and incubated for 4 hours. In some experiments, the target cells were incubated with an anti-HLA class I framework MoAb (w6/32; American Type Culture Collection, Manassas, VA) or an anti-HLA-DR MoAb (L243; American Type Culture Collection) at an optimal concentration (10 $\mu\text{g}/\text{mL}$) for 30 minutes before adding effector cells to determine whether cytotoxicity was restricted by HLA class I. To determine whether WT1-specific CTLs lyse myeloma cells via recognition of the WT1 peptide, which is naturally processed in myeloma cells and expressed in the context of HLA-A24, cold target inhibition assay was performed. WT1 peptide-loaded and unloaded autologous LCL or HLA-A24-positive leukemia cell line MEG01, which was shown to be lysed by WT1-specific CTLs in a WT1-specific manner, was used as cold target cells. After incubation for 4 hours, 100 μL of supernatant were collected from each well. The percentage of specific lysis was calculated as follows: (experimental release cpm - spontaneous release cpm)/(maximal release cpm - spontaneous release cpm). Cytotoxicity mediated by purified perforin was measured

by using 2-hour ^{51}Cr release assays and the trypan blue exclusion method. ^{51}Cr -labeled target cells were incubated with various concentrations of purified perforin in the assay buffer [150 mmol/L NaCl, 20 mmol/L HEPES, and 2.5 mmol/L CaCl_2 (pH 7.4)] for 2 hours at 37°C. After incubation, supernatants were harvested after centrifugation of the microtiter plates, and radioactivity was determined. For trypan blue exclusion, the cells incubated with or without purified perforin were stained with trypan blue, and the percentages of stained cells were determined.

Inhibition of Perforin-Mediated Cytotoxicity. To examine the Ca^{2+} dependency of the cytotoxicity, cytotoxicity assays were performed in the presence of EGTA (Sigma, St. Louis, MO) at various concentrations. To evaluate the role of perforin in CTL-mediated cytotoxicity, effector T cells were pretreated with concanamycin A (CMA; Wako Pure Chemical Industries, Osaka, Japan) at various concentrations for 2 hours and then incubated with the target cells in the presence of CMA. CMA is an inhibitor of vacuolar type H^+ -ATPase that inhibits perforin-based cytotoxicity, mostly by accelerated degradation of perforin caused by an increase in the pH of lytic granules (27). Treatment with CMA at the concentration used in the present study showed no toxic effect against T lymphocytes and myeloma cells as determined from cell growth curves and ^{51}Cr release assays (data not shown).

Detection of Interferon- γ Production. The response of WT1-specific CTLs to various stimulator cells was examined by determining IFN- γ production. For the assays of IFN- γ production, 1×10^5 WT1-specific CTL clone cells or bulk CTLs and 5×10^4 MMC-treated tumor cells were suspended in 200 μL of RPMI 1640 supplemented with 10% FCS and cultured in flat-bottomed microtiter wells in the presence of 10 units/mL recombinant human IL-2. After 72 hours, the supernatants were collected from each well and assayed for IFN- γ production by ELISA.

ELISPOT Assays. ELISPOT assays were performed as described previously (28). Briefly, 96-well flat-bottomed MultiScreen-HA plates with a nitrocellulose base (Millipore; Millipore Corp., Bedford, MA) were coated with 10 $\mu\text{g}/\text{mL}$ anti-IFN- γ MoAb (R&D Systems, Minneapolis, MN) and incubated overnight at 4°C. After washing with PBS, the plates were blocked with the culture medium for 1 hour at 37°C. T2-A24 cells (5×10^4 cells) were pulsed with 10 $\mu\text{mol}/\text{L}$ WT1-T2 peptide or PBS alone in RPMI 1640 with 10% FCS for 1 hour at room temperature, and then responder cells were seeded in each well. The plates were incubated in a 5% CO_2 incubator at 37°C for 20 hours and washed extensively with PBS containing 0.05% Tween 20. A polyclonal rabbit anti-IFN- γ antibody (Endgen, Woburn, MA) was added to individual wells and left for 90 minutes at room temperature, followed by exposure to peroxidase-conjugated goat antirabbit IgG (Zymed, San Francisco, CA) for an additional 90 minutes. For visualization of IFN- γ -specific spots, 100 μL of 0.1 mol/L sodium acetate buffer (pH 5.0) containing 3-amino-9-ethylcarbazole (Sigma) and 0.015% H_2O_2 were added to each well. After 40 minutes, the reaction was stopped by washing with water, and the plates were dried. Diffuse large spots were counted under a dissecting microscope.

Tetramer Production and Staining. HLA-A24/WT1 peptide tetramer was produced as described previously (28). Briefly, BL21(DE3) pLysS (Novagen, Madison, WI) competent cells were transformed with plasmid pET11d (Novagen) encoding HLA-A*2402 heavy chain or plasmid pET-3a (Novagen) encoding β_2 -microglobulin to produce the recombinant proteins. Expression of the HLA heavy chain was limited to the extracellular domain, and the COOH terminus of the domain was modified by the addition of a substrate sequence for the biotinylating enzyme BirA. Monomeric HLA-peptide complexes were folded by adding the HLA protein to β_2 -microglobulin in the presence of the modified 9-mer WT1-T2 peptide (CYTWNQMNL) or HIV-1 Env (RYLRDQQLL). Proteins were dialyzed against water and then concentrated. After purification by gel filtration, the complex was biotinylated using recombinant BirA enzyme (Avidity, Denver, CO) and then purified by gel filtration. HLA-peptide tetramers were made by mixing the biotinylated HLA with phycoerythrin-labeled streptavidin (Molecular Probes, Eugene, OR) at a molar ratio of 4:1. Tetramers were purified by gel filtration on a Superdex 200 HR 10/30 (Amersham Pharmacia Biotech, Uppsala, Sweden) and stored at 4°C until use. CTLs were stained with a mixture of the tetramer at 0.1 mg/mL and FITC-conjugated anti-CD8 MoAb (BD PharMingen) at 4°C for 20 minutes. After washing twice, the stained cells were analyzed with a flow cytometer (FACScan; Becton Dickinson).

Statistical Analysis. Statistical evaluation was performed with the StatView 5.0 statistical software (Abacus Concept, Berkeley, CA). Differences in the expression of WT1 mRNA in leukemia, myeloma, and lymphoma cells; surface HLA class I molecules on myeloma and lymphoma cells; and perforin-mediated cytotoxicity against myeloma and lymphoma cells were compared using the Mann-Whitney *U* test for parallel groups. *P* values of <0.05 were considered statistically significant.

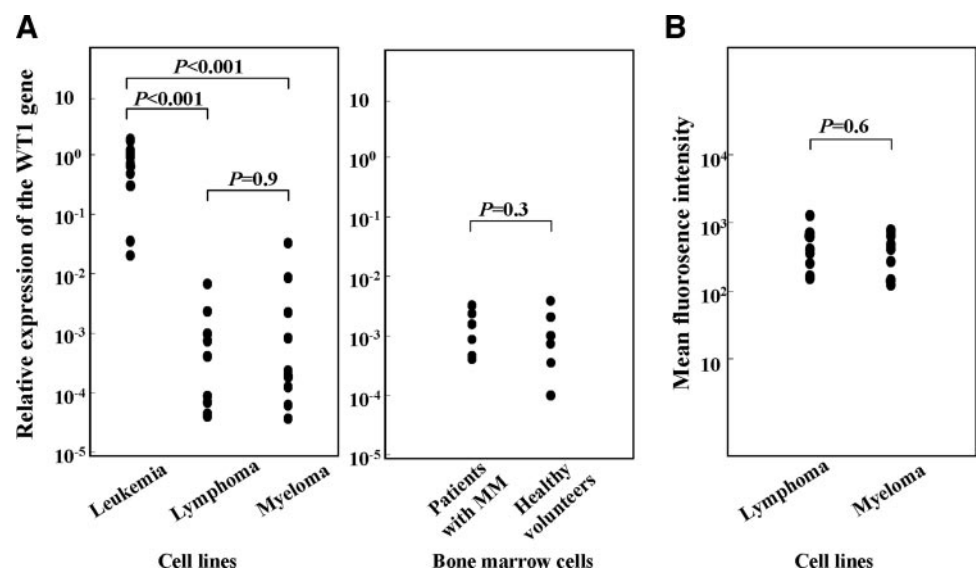
RESULTS

Expression of WT1 Messenger RNA and HLA Class I Molecules. WT1 mRNA expression levels in the human leukemia, myeloma, lymphoma cell lines, and bone marrow cells was determined by quantitative RT-PCR and calculated relative to that in the human leukemia cell line K562. Because relative WT1 expression levels in most normal tissues are $<10^{-6}$, levels of $>10^{-5}$ were considered positive. As shown in Fig. 1A, the relative WT1 expression levels in leukemia cell lines were 1.9×10^{-2} to 2.2×10^0 . These values are significantly higher than those in myeloma and lymphoma cell lines [3.6×10^{-5} to 3.1×10^{-2} ($P < 0.001$) and 3.8×10^{-5} to 6.3×10^{-3} ($P < 0.001$), respectively]. No difference in WT1 expression levels between myeloma and lymphoma cell lines was detected ($P = 0.9$). The relative WT1 expression levels in bone marrow cells isolated from patients with MM and healthy volunteers were 4.9×10^{-4} to 3.9×10^{-3} and 1.2×10^{-4} to 4.7×10^{-3} , respectively, and no difference in WT1 expression levels between these two groups was detected ($P = 0.3$).

The surface expression of HLA class I on myeloma and lymphoma cell lines was determined by flow cytometric analysis as shown in Fig. 1B. All of the cell lines examined expressed surface HLA class I molecules, and the mean fluorescence intensities corresponding to HLA class I molecules on myeloma and lymphoma cell lines were not significantly different ($P = 0.6$).

Cytotoxicity of WT1-Specific Cytotoxic T Lymphocytes against Myeloma Cells and Lymphoma Cells. We previously established a WT1-T2 peptide-specific and HLA-A24-restricted CTL clone, designated TAK-1 (16). The TAK-1 clone cells had been stored frozen in liquid nitrogen and were thawed for use in the present study. To confirm that the freezing and thawing procedures had not affected the antigen specificity and HLA restriction of the TAK-1 cells, we first investigated their cytotoxic activity against peptide-loaded and unloaded cells. TAK-1 lysed autologous LCLs that had been loaded with the WT1-T2 peptide but was not cytotoxic to unloaded LCLs or to

Fig. 1 Expression levels of WT1 mRNA and surface HLA class I molecules. **A.** Expression levels of WT1 mRNA in various leukemia ($N = 15$), lymphoma ($N = 10$), and myeloma ($N = 10$) cell lines; bone marrow cells isolated from patients with MM ($N = 6$); and healthy volunteers ($N = 6$) were determined by quantitative RT-PCR as described in Materials and Methods. **B.** Expression levels of surface HLA class I molecules on various lymphoma ($N = 10$) and myeloma ($N = 10$) cell lines were determined by flow cytometry.



those loaded with other WT1-derived peptides (data not shown). TAK-1 appeared to be cytotoxic only to HLA-A24–positive allogeneic LCLs and the HLA-A*2402 transfectant cell line T2-A24 (but not to its parent cell line, T2) in the presence of WT1-T2 peptide, as demonstrated previously. These data confirmed that TAK-1–mediated cytotoxicity is WT1-T2 peptide-specific and restricted by HLA-A24. The cytotoxicity of TAK-1 against the leukemia, lymphoma, and myeloma cell lines is shown in Table 1. As shown previously, TAK-1 exerted cyto-

Table 1 Cytotoxicity of WT1-specific CTLs against various target cells

Target cells	HLA-A24	% of specific lysis*			% of myeloma cells†
		20:1	10:1	5:1	
Leukemia cell lines					
MEG01	+	48.3	35.8	28.3	
TK91	+	41.8	35.1	20.9	
K562	–	3.7	3.3	1.3	
NB4	–	5.0	3.3	2.8	
Lymphoma cell lines					
NAK	+	5.8	4.1	2.0	
IZU	+	3.6	2.6	1.4	
KUB	+	4.8	3.3	2.1	
DHL8	+	1.5	1.2	0.2	
Daudi	–	0.1	0.1	0.1	
Raji	–	1.0	0.3	0.0	
Namalwa	–	0.4	0.0	0.0	
Myeloma cell lines					
KMS-21BM	+	56.0	55.5	42.4	
KMS-34	+	61.9	58.8	50.9	
KMS-28BM	+	53.7	47.2	43.6	
KMS-18	+	38.9	33.0	13.5	
KMM-1	+	29.9	21.3	12.9	
KMS-11	+	27.2	24.5	15.8	
KMS-12PE	–	3.9	3.7	3.3	
KMS-20	–	3.0	3.4	2.0	
KMS-26	–	3.5	2.6	2.4	
Bone marrow cells isolated from MM patients					
Patient 1	+	22.3	19.7	13.7	78.3
Patient 2	+	21.8	15.0	11.5	Unknown
Patient 3	+	27.9	17.6	6.4	72.1
Patient 4	+	21.2	14.7	4.9	67.3
Patient 5	+	13.8	9.8	9.6	69.7
Patient 6	+	15.6	10.8	9.3	32.4
Patient 7	+	20.5	19.1	15.9	26.4
Patient 8	+	16.4	12.1	9.3	27.0
Patient 9	–	2.6	0.8	0.0	30.3
Patient 10	–	2.0	0.0	0.2	83.6
Patient 11	–	0.0	0.0	0.0	76.0
Bone marrow cells isolated from healthy volunteers					
Donor 1	+	0.9	0.8	0.5	
Donor 2	+	0.0	0.0	0.0	
Donor 3	+	1.1	0.4	0.0	
Donor 4	+	0.0	0.0	0.0	
Purified normal CD34 ⁺ cells					
Donor 1	+	0.0	0.0	0.0	
Donor 2	+	0.0	0.0	0.0	

* The cytotoxicity of WT1 peptide-specific CTL clone TAK-1 against the various HLA-A24–positive and –negative cell lines, bone marrow cells isolated from patients with MM and healthy volunteers, and CD34⁺ cells isolated from cord blood was determined by 4-hour ⁵¹Cr-release assays at effector to target ratios of 20:1, 10:1, and 5:1.

† The percentages of myeloma cells contained in whole bone marrow cells of patients with MM were shown.

toxicity against HLA-A24–positive leukemia cell lines that expressed WT1 abundantly, whereas no cytotoxicity against HLA-A24–negative leukemia cell lines was detected. TAK-1 appeared to have no cytotoxic activity against lymphoma cell lines that expressed WT1 at a low level, regardless of their HLA-A24 expression status. In contrast, TAK-1 exhibited strong cytotoxicity against HLA-A24–positive myeloma cell lines but not against HLA-A24–negative cells, although WT1 expression levels in myeloma cells and lymphoma cells were almost the same. The cytotoxicity of TAK-1 against bone marrow cells freshly isolated from patients with MM and healthy volunteers is also shown in Table 1. As observed with the myeloma cell lines, TAK-1 was cytotoxic to myeloma cells isolated from HLA-A24–positive patients. Although WT1 expression levels in bone marrow cells isolated from the healthy individuals and the patients with MM were almost the same, TAK-1 was not cytotoxic to normal bone marrow cells. Furthermore, TAK-1 did not appear to be cytotoxic to purified normal CD34⁺ hematopoietic progenitor cells.

To confirm that the cytotoxicity of TAK-1 against myeloma cells is restricted by HLA-A24, inhibition assays using anti-HLA MoAbs were performed. As shown in Fig. 2A, the addition of an anti-HLA class I framework MoAb, but not an anti-HLA-DR MoAb, resulted in inhibition of the cytotoxicity mediated by TAK-1 against HLA-A24–positive myeloma cells. Taken together with the data shown in Table 1, these findings demonstrate that the cytotoxicity of TAK-1 against myeloma cells is restricted by HLA-A24.

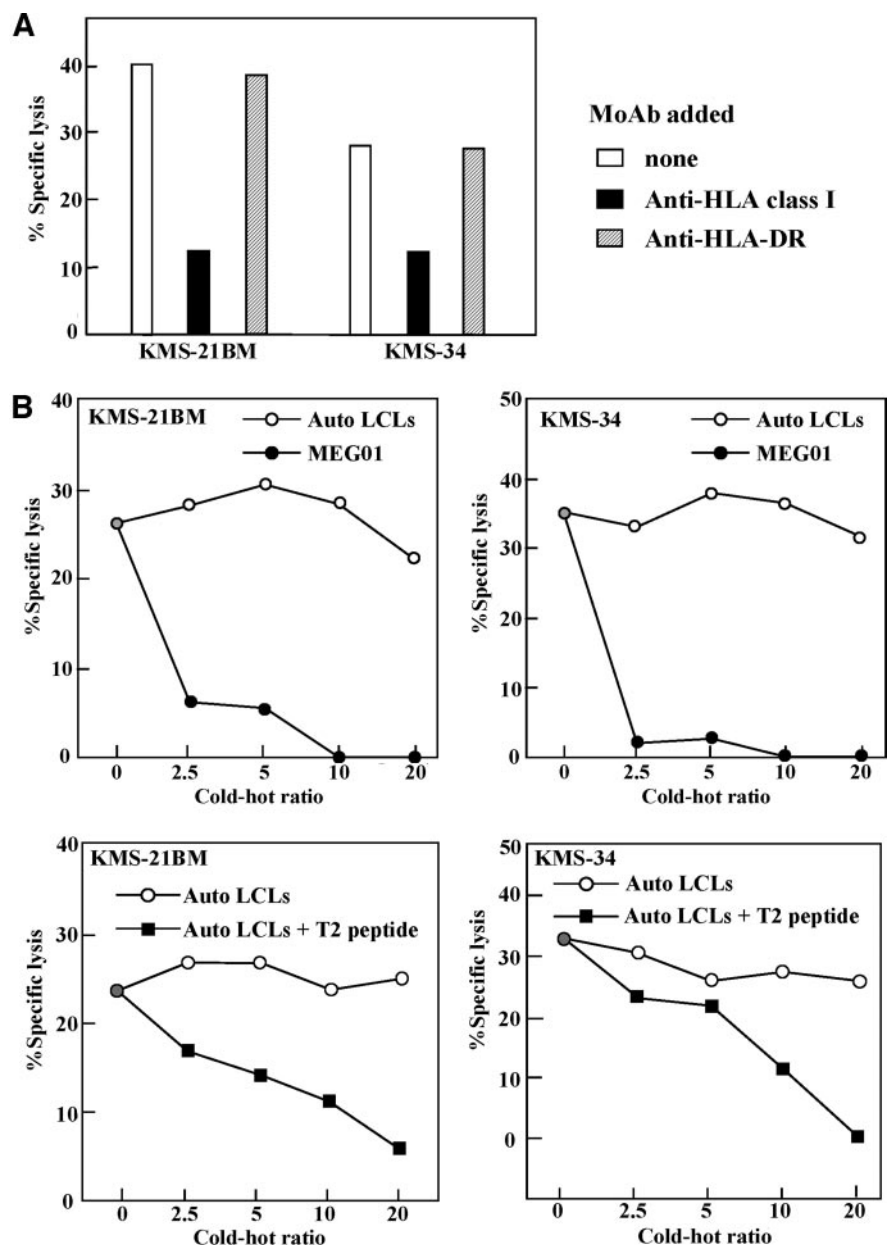
To confirm that the cytotoxicity of TAK-1 against myeloma cells was mediated by specific recognition of the endogenously processed WT1, we performed cold target inhibition experiments. MEG01 is a leukemia cell line that has been previously shown by an antisense oligonucleotide method to be lysed by TAK-1 in a WT1-specific and HLA-A24–restricted manner (16). As shown in Fig. 2B, the addition of radioisotope-unlabeled MEG01 markedly decreased TAK-1–induced ⁵¹Cr release from two myeloma cell lines, KMS-21BM and KMS-34, whereas the addition of unlabeled autologous LCL cells had no effect on cytotoxicity. Similarly, the addition of unlabeled WT1-T2 peptide-loaded autologous LCL cells decreased the cytotoxicity of TAK-1 against myeloma cell lines, as shown in Fig. 2B. These findings strongly suggest that WT1 is naturally processed in myeloma cells, expressed in the context of HLA-A24, and recognized by WT1-specific CD8⁺ CTLs.

Granule Exocytosis Pathway of Cytotoxicity against Myeloma Cells Mediated by WT1-Specific Cytotoxic T Lymphocytes.

We next examined the cytotoxic pathway of WT1-specific CTLs against myeloma cells. The perforin/granzyme pathway is known to be Ca²⁺ dependent, and recent studies have shown that extracellular Ca²⁺ is also necessary for the Fas/Fas ligand system (29). According to these findings, the cytotoxic activity of TAK-1 was examined in the absence of extracellular Ca²⁺. As shown in Fig. 3A, the cytotoxicity of TAK-1 against myeloma cells was dramatically decreased in the presence of the Ca²⁺-chelating agent EGTA. Thus, TAK-1-mediated cytotoxicity appears to be Ca²⁺ dependent.

Next, the significance of the granule exocytosis pathway was examined with an inhibitor of vacuolar type H⁺-ATPase, CMA. Recent studies have shown that CMA is a selective

Fig. 2 HLA restriction and antigen specificity of cytotoxicity mediated by TAK-1 against myeloma cells. **A**, Inhibition of TAK-1-mediated cytotoxicity against myeloma cells by a HLA class I MoAb. The cytotoxicity of TAK-1 against the HLA-A24-positive myeloma cell lines KMS-21BM and KMS-34 was determined in the presence or absence of anti-HLA class I framework MoAb or anti-HLA-DR MoAb at an effector to target ratio of 10:1. The results shown represent the means of triplicate experiments. **B**, Cold target inhibition assays. ^{51}Cr -labeled KMS-21BM and KMS-34 myeloma cells (1×10^4 cells) were mixed with various numbers of unlabeled autologous LCLs or MEG01 HLA-A24-positive leukemia cells, which have previously been reported to be lysed by TAK-1 in a WT1-specific manner. ^{51}Cr -labeled KMS-21BM and KMS-34 myeloma cells (1×10^4 cells) were also mixed with various numbers of unlabeled autologous LCLs or with WT1-T2 peptide-loaded autologous LCLs. The cytotoxicity of TAK-1 against the mixture of ^{51}Cr -labeled and unlabeled target cells was determined by 4-hour ^{51}Cr release assays at an effector to ^{51}Cr -labeled target cell ratio of 10:1. The results shown represent the means of triplicate experiments.



inhibitor that blocks perforin-based cytotoxicity, mostly as a result of accelerated degradation of perforin by an increase in the pH of lytic granules, but does not affect Fas-dependent cytotoxicity (27). The results shown in Fig. 3B demonstrate that pretreatment of TAK-1 with CMA at concentrations of >10 nmol/L resulted in inhibition of TAK-1 cytotoxicity against both KMS-21BM and KMS-34. These data suggest that the cytotoxicity of WT1-specific CTLs against myeloma cells is mediated through the granule exocytosis pathway.

High Sensitivity of Myeloma Cells to Perforin-Mediated Cytotoxicity. Because the expression levels of surface HLA class I molecules on myeloma cells and lymphoma cells appeared to be almost the same, the amounts of the WT1 peptide/HLA-A24 complex on myeloma cells and lymphoma cells after

the addition of a WT1 peptide exogenously at the same concentration should also be equivalent. Therefore, comparison of the cytotoxic activities of WT1 peptide-specific CTLs against target cells loaded with a certain concentration of WT1 peptide may be a valid approach to determining the relative sensitivity of each target cell to perforin-mediated cytotoxicity. Accordingly, we compared the cytotoxic activities of TAK-1 against WT1 peptide-loaded myeloma cells and lymphoma cells at a low effector to target ratio. As shown in Fig. 4, TAK-1 was not cytotoxic to lymphoma cells loaded with WT1-T2 peptide at low concentrations; however, at these low concentrations, myeloma cells were lysed in a dose-dependent manner. At high concentrations of WT1 peptide, lymphoma cells were also lysed by TAK-1, although cytotoxic activity against WT1 peptide-loaded my-

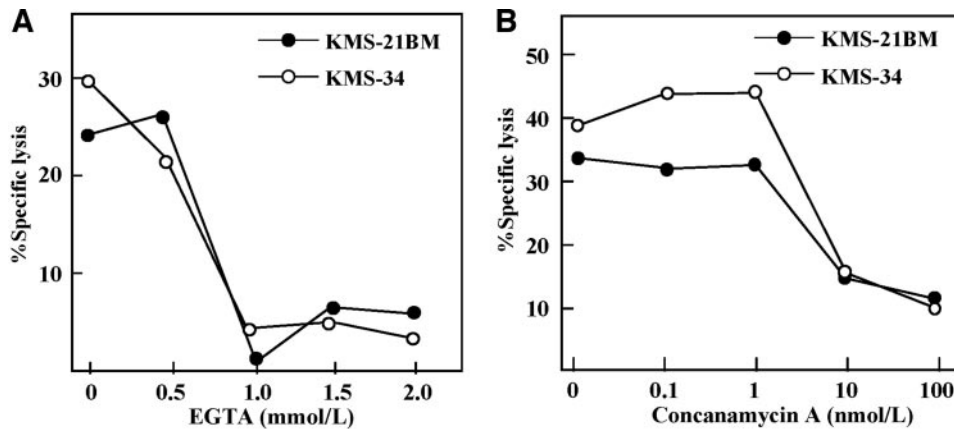


Fig. 3 Effects of EGTA and CMA on cytotoxicity of TAK-1 against myeloma cells. The cytotoxicity of TAK-1 against the HLA-A24-positive myeloma cell lines KMS-21BM and KMS-34 in the presence of EGTA or CMA at various concentrations was determined by 4-hour ^{51}Cr release assays at an effector to target cell ratio of 10:1. The results shown represent the means of triplicate experiments.

eloma cells was higher than that against WT1 peptide-loaded lymphoma cells. These data strongly suggest that myeloma cells are highly sensitive to the granule exocytosis pathway of WT1-specific CTLs.

We further compared the sensitivities of myeloma cells and lymphoma cells to perforin-mediated cytotoxicity by using purified perforin. As shown in Fig. 5, both the ^{51}Cr release assay and the trypan blue exclusion test revealed that myeloma cells are significantly more sensitive than lymphoma cells to cytotoxicity mediated by purified perforin.

Interferon- γ Production by WT1-Specific Cytotoxic T Lymphocytes Recognizing Myeloma Cells and Lymphoma Cells. We addressed the question of whether WT1-specific CTLs can recognize WT1 peptide/HLA-A24 complexes expressed on myeloma cells and lymphoma cells and produce

cytokines equivalently in response to both types of malignant cell. To investigate this point, TAK-1 was stimulated with myeloma cells and lymphoma cells, and then IFN- γ production by TAK-1 was measured. As shown in Fig. 6, TAK-1 secreted IFN- γ equivalently in response to stimulation with HLA-A24-positive lymphoma cells and HLA-A24-positive myeloma cells; however, IFN- γ production by TAK-1 was not detectable after coculture with HLA-A24-negative cells. These data strongly suggest that although the production level of WT1 protein in myeloma cells and lymphoma cells is relatively low compared with that in acute leukemia cells, WT1 is efficiently processed in these mature B-cell malignant cells, and that WT1-specific CTLs can react equivalently to lymphoma cells and myeloma cells through the recognition of WT1 peptide in the context of HLA molecules.

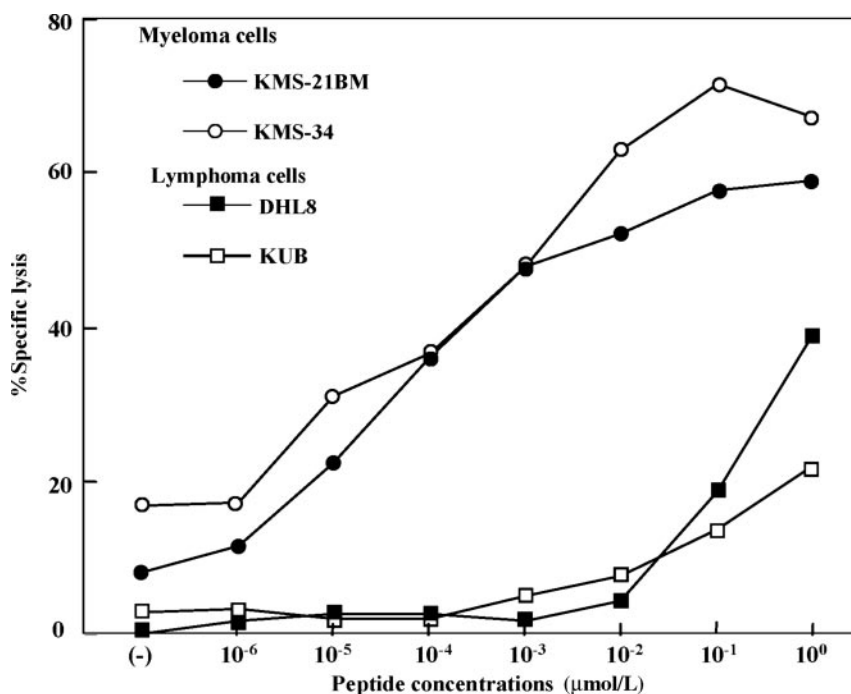
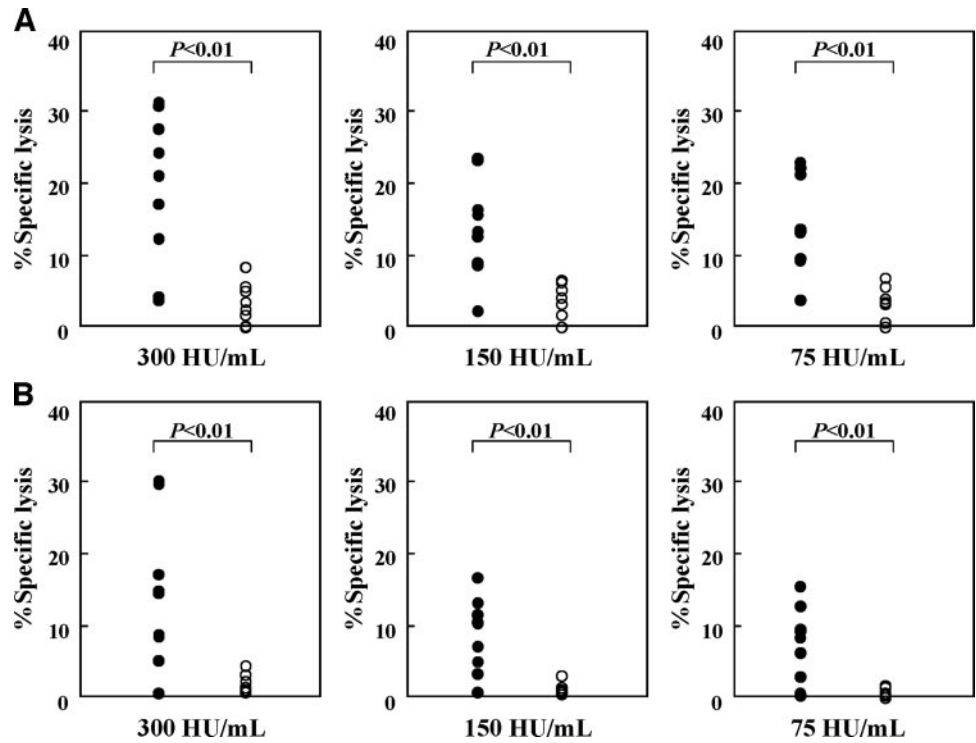


Fig. 4 Effect of WT1 peptide concentration on cytotoxicity of TAK-1 against myeloma cells and lymphoma cells. The cytotoxicity of TAK-1 against myeloma cell lines KMS-21BM and KMS-34 and lymphoma cell lines DHL8 and KUB, loaded with various concentrations of WT1-T2 peptide for 1 hour, was determined by 4-hour ^{51}Cr release assays at an effector to target ratio of 2:1. The results shown represent the means of triplicate experiments.

Fig. 5 Cytotoxicity of myeloma and lymphoma cells induced by purified perforin. **A**, trypan blue exclusion test. The myeloma cell lines (●; *n* = 10) and lymphoma cell lines (○; *n* = 10) were incubated with various concentrations of purified perforin for 2 hours at 37°C and then stained with trypan blue, and the percentages of stained cells were determined. **B**, ⁵¹Cr release assay. ⁵¹Cr-labeled myeloma cell lines (●) and lymphoma cell lines (○) were incubated with various concentrations of purified perforin. After 2 hours of incubation at 37°C, supernatants were harvested after centrifugation of the microtiter plates, and radioactivity was determined. The percentage of specific lysis was calculated as described in Materials and Methods. Each result represents the mean of triplicate experiments.



Generation of WT1 Peptide-Specific Cytotoxic T Lymphocytes from Patients with Multiple Myeloma. Finally, to investigate the feasibility of cellular immunotherapy for MM targeting WT1, we examined whether WT1 peptide-specific CTL precursors were present in the patients with MM. For detection of a small number of WT1 peptide-specific CTLs, we performed ELISPOT assays as described above. A T-cell response was considered positive if the number of spots in the presence of WT1 peptide-loaded T2-A24 cells was ≥ 3 -fold

higher than that in the presence of non-WT1 peptide-loaded T2-A24 cells and if there was a minimum of 20 peptide-specific spots in each well (after subtracting the number of spots observed in the presence of non-WT1 peptide-loaded T2-A24 cells). WT1 peptide-specific responses were detected in all five patients with MM and three healthy volunteers as shown in Fig. 7A. The percentage of positive wells in cultures of lymphocytes from the five patients with MM and three healthy volunteers ranged from 2.1% to 13.3% and from 4.2% to 12.5%, respec-

Stimulator cells HLA-A24

Myeloma cells

- KMS-21BM +
- KMS-34 +
- KMS-12PE -
- KMS-26 -

Lymphoma cells

- DHL8 +
- KUB +
- Raji -
- Namalwa -

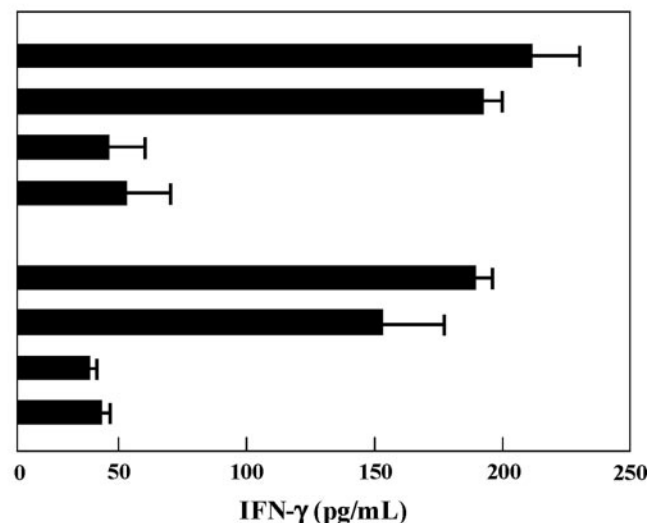


Fig. 6 Recognition of myeloma and lymphoma cells by TAK-1. TAK-1 cells were cocultured with various MMC-treated cell lines. After 3 days, the culture supernatants were harvested and assayed by ELISA for IFN- γ production. The data are expressed as the mean counts \pm SDs of three wells.

tively, suggesting that there is no significant difference in the frequency of WT1-specific CTL precursors between patients with MM and healthy individuals.

To address whether WT1 peptide-specific CTLs generated from patients with MM can recognize WT1 peptide/HLA-A24 complexes expressed on myeloma cells and can lyse myeloma cells, IFN- γ production and cytotoxicity against myeloma cells were determined. As shown in Fig. 7B, WT1 peptide-specific bulk #3-B4 CTLs secreted IFN- γ in response to stimulation with HLA-A24-positive myeloma cells and WT1 peptide-loaded T2-A24 cells; however, IFN- γ production by #3-B4 CTLs was not detectable after coculture with HLA-A24-negative myeloma cells or non-WT1 peptide-loaded T2-A24 cells. Furthermore, as shown in Fig. 7C, #3-B4 CTLs exerted cytotoxicity against HLA-A24-positive myeloma cells and WT1 peptide-loaded T2-A24 cells. The #3-B4 CTLs were expanded by additional stimulation with MMC-treated WT1-T2 peptide-loaded T2-A24 cells. As shown in Fig. 7D, most #3-B4 CTLs were stained with WT1/HLA-A24 tetramer. These data strongly suggest that WT1-specific CTL

precursors are indeed present in patients with MM and that immunotherapy for MM targeting WT1 might be feasible.

DISCUSSION

The new findings obtained from the present series of experiments are as follows. First, the expression level of WT1 mRNA in myeloma cells is relatively low compared with that in acute leukemia cells and is similar to that in B-lymphoma cells. Second, although the expression levels of WT1 in myeloma cells and lymphoma cells and surface HLA class I on myeloma cells and lymphoma cells are almost the same, only myeloma cells are lysed efficiently by WT1-specific CTLs in a HLA class I-restricted manner. Third, the cytotoxic pathway of WT1-specific CTLs against myeloma cells appears to be the conventional perforin-dependent granule exocytosis, and WT1 peptide-loaded myeloma cells are more susceptible to lysis by WT1-specific CTLs compared with WT1 peptide-loaded lymphoma cells. Fourth, WT1-specific CTLs produce IFN- γ at almost the

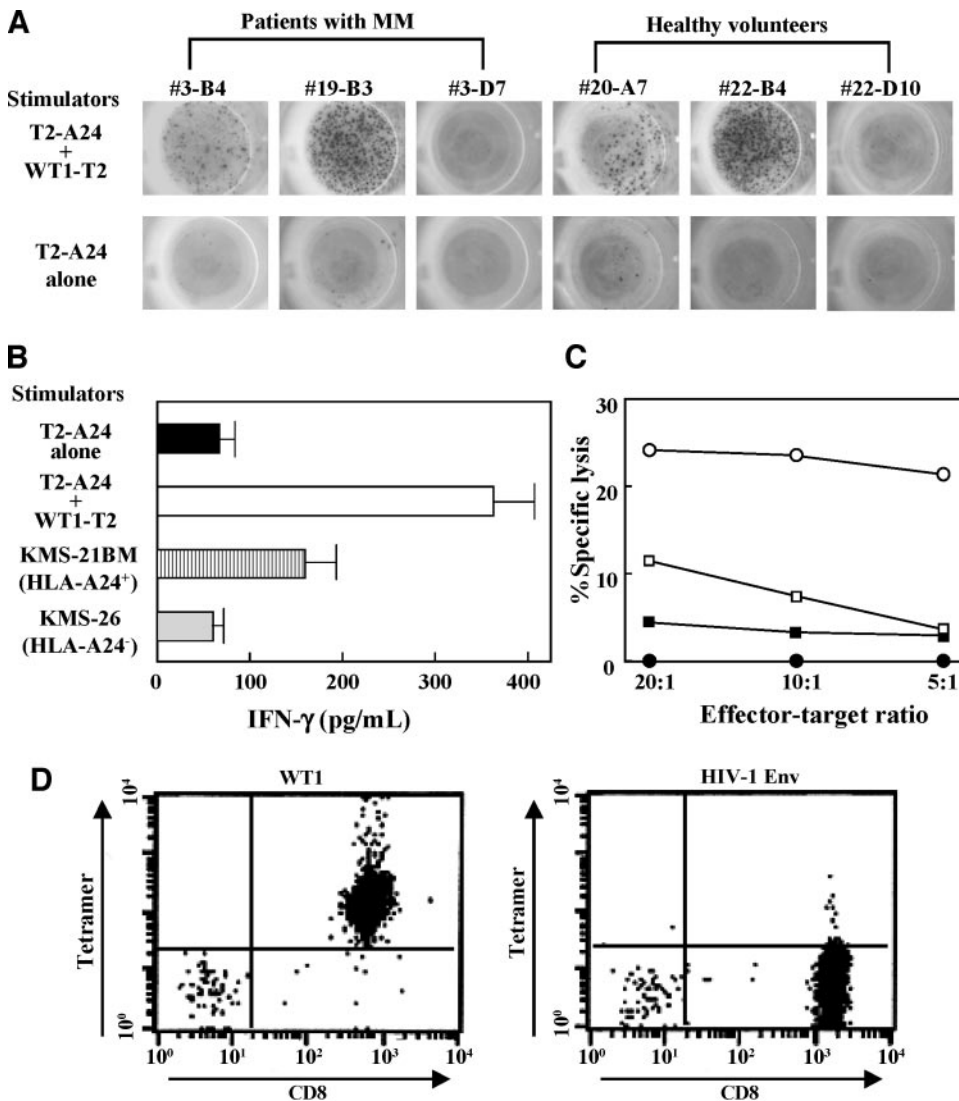


Fig. 7 Generation of WT1-specific CTLs from the patients with MM. A, ELISPOT assays. CTLs generated after the third stimulation with WT1-T2 peptide were tested for their specificity. ELISPOT assays were performed by incubating the CTLs with T2-A24 cells pulsed with 10 μ Mol/L WT1-T2 peptide or T2-A24 cells alone. These figures show examples of results considered positive (#3-B4, #19-B3, #20-A7, and #22-B4) or negative (#3-D7 and #22-D10). B, ELISA for IFN- γ production. Bulk #3-B4 CTLs were cocultured with various MMC-treated cells with or without WT1-T2 peptide. After 3 days, the culture supernatants were harvested and assayed by ELISA for IFN- γ production. The data are expressed as the mean counts \pm SDs of three wells. C, 51 Cr release assay. The cytotoxicity of the bulk #3-B4 CTLs against target cells, T2-A24 cells loaded with WT1-T2 peptide (○), T2-A24 cells alone (●), KMS-21BM (□), and KMS-26 (■) was determined by 4-hour 51 Cr release assays at various effector to target ratios. The results shown represent the means of triplicate experiments. D, tetramer staining. CTLs were stained with phycoerythrin-labeled tetramer in combination with FITC-conjugated anti-CD8 MoAb. #3-B4 CTLs were stained by the A*2402/WT1-T2 tetramer, but not by the irrelevant A*2402/HIV-1 env.

same level in response to stimulation with myeloma cells and lymphoma cells, suggesting that WT1 is processed and expressed similarly in the context of HLA class I molecules in myeloma cells and lymphoma cells. Fifth, and most importantly, the sensitivity of myeloma cells to cytotoxicity induced by purified perforin is significantly higher than that of lymphoma cells. In addition, WT1-specific CTL precursors appeared to be present in patients with MM as well as in healthy individuals.

Recently, various types of tumor-associated antigens have been identified. Some of them, including fusion gene products such as BCR-ABL (30–35) and ETV6-AML1 (36), are undoubtedly expressed only in tumor cells. However, the distribution of many tumor-associated antigens identified thus far is not strictly restricted to malignant cells, and normal cells also express these antigens at a relatively low level. The reason why only tumor cells and not normal cells are lysed by antigen-specific CTLs has been thought to be that the complex of tumor-associated antigen-derived peptide and HLA molecule is expressed on normal cells at too low a level to be recognized by CTLs. However, a previous study has revealed that a single peptide/major histocompatibility complex expressed on target cells can elicit a CTL response (37). These findings strongly suggest that protective mechanisms against CTL-mediated cytotoxicity must be present in normal cells, but the precise mechanism of this phenomenon is still obscure.

It is well known that CTLs are resistant to perforin-mediated cytotoxicity, for if they were susceptible, they would be killed by the perforin that they themselves release. Therefore, clarifying the mechanism of CTL resistance to perforin-mediated cytotoxicity might provide an insight into the cause of differential sensitivity to WT1-specific CTL-mediated cytotoxicity between myeloma cells and lymphoma cells. Previous studies have revealed that protective molecules specifically expressed on CTLs interact with perforin, thereby rendering CTLs resistant to perforin-mediated cytotoxicity (38, 39); however, the precise mechanism of the interaction between perforin and these lymphocyte membrane proteins is still unknown. Recently, it has been reported that proteolysis of perforin by surface cathepsin B provides self-protection to CTLs (40). The other molecular model that has been proposed for CTL self-protection involves the serpin granzyme B inhibitor PI-9 (41). Although these molecules were not studied here, it is possible that different expression levels of these inhibitors of perforin and granzymes determine the sensitivity of tumor cells to perforin-mediated cytotoxicity.

Resistance to perforin-mediated cytotoxicity possibly induced by membrane-stabilizing mechanisms has also been shown in human cytomegalovirus-infected fibroblasts (42). In addition, the human leukemia cell line ML-2 has been reported to be recognized by NK cells but resistant to NK cell-mediated cytotoxicity because of defective perforin binding (43). In that study, the binding of perforin to the leukemia cell membrane was examined by flow cytometry. However, it was argued that flow cytometry cannot assess surface binding of perforin to target cells (44). Indeed, binding of perforin to the cell membrane of target cells that are susceptible to granule exocytosis could not be detected by flow cytometry in our study (data not shown). Therefore, it will be necessary to use other experimental systems to clarify the precise mechanisms of susceptibility to perforin-mediated cytotoxicity.

Because the clinical outcomes of conventional chemotherapy for MM are not satisfactory, novel therapeutic approaches, including cellular immunotherapy, have been proposed. Although some potential target antigens for immunotherapy of MM have been identified, including idiotype (45–47), MUC1 (48), sperm protein 17 (49, 50), SPAN-Xb (51), and MAGE families (52), the number of suitable target antigens recognized by CTLs directed against myeloma cells is still limited (53). To the best of our knowledge, this is the first report to describe the efficacy of WT1-specific CTLs against MM. The present findings may contribute to the development of novel immunotherapeutic strategies for MM and suggest that vaccination with a WT1-derived peptide or WT1-coding DNA and adoptive immunotherapy using WT1-specific CTLs may provide an effective treatment option for MM as well as acute leukemia.

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